



Research Article

ATTENUATION OF GROWTH OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN RESPONSE TO SILVER NANOPARTICLES

Sumayah Abdulhussien Ibraheem ^{1*}, Al-keline Haitham Ali Kadhem ¹, Nada H. A. Al-Mudallal ², Ihsan Ali Kadhim ³, Mihaileescu Dan Florin ¹

¹Faculty of biology/university Bucharest/ Romania

²Al Iraqi university college of medicine /medical microbiology department/Baghdad/Iraq

³Ministry of science and Technology/ Industrial development and research

*Corresponding Author Email: sumayahibraheem8@gmail.com

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ABSTRACT

Nanotechnology is emerging as a new interdisciplinary field combining microbiology, Chemistry, physics, and material science. Recent advances promise developments in the synthesis, modification and practical applications of nanoparticles (NPs). Nanoparticles were excellent antibacterial agents with potential clinical applications. Silver Nanoparticles have been successfully used in a wide range of applications including wound dressing, protective clothing, antibacterial surfaces, food preservation, and cosmetics as biocidal and disinfecting agents. The aim of this study was to investigate the mechanism of silver nanoparticle action against methicillin-resistant *Staphylococcus aureus*. The Gram-positive methicillin-resistant *Staphylococcus aureus* were used to evaluate the antibacterial activities of silver nanoparticles (Ag NPs). The growth rate of methicillin-resistant *Staphylococcus aureus* was investigated under varying Ag NPs concentrations by scanning electron microscope (SEM) & acridine orange /ethidium bromide (AO-EtBr) staining. The Quantitative Real-time PCR experiment showed that the *mecA* gene from the bacterial cells treated with Ag-NPs was downregulated compared to that in the untreated cells.

Keywords: methicillin-resistant, *Staphylococcus aureus*, silver nanoparticles, *mecA* gene

INTRODUCTION

Nanotechnology can actually be defined as the technology at the dimensions of 1-billionth of a meter. It's the design, characterization, synthesis and applications of substances, systems, devices and systems via controlling form and size at nanometer scale. This science has a great value of modern research in the major field of biology, chemistry, physics and materials sciences ¹. It is noted that physical and chemical properties change when decrease the particle size to nanoscale ². Nanoparticles (NPs) can be classified into different classes based on their properties, shapes or sizes. The different groups include fullerenes, metal NPs, ceramic NPs, and polymeric NPs. NPs possess unique physical and chemical properties due to their high surface area and nanoscale size. Generally, metal nanoparticles can be synthesized and stabilized by chemical, physical and biological methods; the chemical approach, such as electrochemical reduction, photochemical reduction and heat evaporation, the physical methods by either evaporation - condensation method or laser ablation method Living organisms have huge potential for the production of nanoparticles /nanodevices of wide applications³. Silver nanoparticles are of hobby due to the precise properties (e.g., Diameter and shape relying optical, electric, and magnetic residences) which may be integrated into antimicrobial applications, biosensor substances, composite fibers, and digital additives Currently most of the applications of the silver nanoparticles are in antibacterial/antifungal agents in biotechnology and bioengineering, textile, water treatment, and silver-based consumer products ⁴. One of the main causes of infection disease is bacteria. Infectious diseases are the leading reason for international morbidity and mortality. The spreading of infectious

diseases is an outcomes from alters in human behavior like lifestyles, land use patterns and wrong utilization of anti-microbial medicine that reason changes in microorganism strains *Staphylococcus aureus* and *E. coli* are typical reasons of varied humans' infections. *Staphylococcus aureus* is a major human pathogen, has a collection of virulence factors and the capability to acquire resistance to most antibiotics ⁵.

Take into account to be a potential "superbug", methicillin-resistant *Staphylococcus aureus* (MRSA) represented one of the main recent infectious pathogens and therefore poses a challenge to hospital infection control⁶. Since the 1960s, methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged, spread globally and come to be an important reason of bacterial infections in both health-care and community sites ⁷. MRSA infection, in otherwise healthy individuals, affects the soft tissues and superficial skin although more serious infections can arise, affecting the deep soft tissues, blood, and bone ⁸. MRSA is able to avoid the body's immune system due to production of biofilm as well as certain toxins ⁹. These virulence factors, in combination with multidrug resistance, cause in high morbidity and mortality rates ¹⁰. The *mecA* gene is a gene found in bacterial cells which allows a bacterium to be resistant to antibiotics such as methicillin, penicillin and other penicillin-like antibiotics. Resistant strains are responsible for many infections originating in hospital. The *mecA* gene encodes the protein PBP2A (penicillin binding protein 2A). PBP2A has a low affinity for beta-lactam antibiotics such as methicillin and penicillin. This enables transpeptidase activity in the presence of beta-lactams, preventing them from inhibiting cell wall synthesis. The *mecA* gene is contained on a mobile gene element, called the staphylococcal cassette chromosome *mec*, from which the gene can undergo horizontal gene transfer and

insert itself into the host species¹¹. This DNA cassette is a 52 kilobase piece of DNA that contains the *mecA* gene, and two recombinase genes, *ccrA* and *ccrB*, which the plasmid uses to insert itself into the genome of the host. These recombinases are essential for the proper insertion of the *mecA* complex into the host genome¹². The *mecA* gene also forms a complex with two regulatory units, *mecI* and *mecR1*. These two genes have the capability to repress *mecA*, deletions or knock-outs in these genes show an increase in resistance of *Staphylococcus aureus* to methicillin. The *S. aureus* strains isolated from humans either lack these regulatory elements, or contain mutations in these genes that cause a loss of function of the protein products that inhibit *mecA*¹³.

MATERIALS AND METHODS

Silver nanoparticles

Silver nanoparticles were obtained from (Sigma, USA), acridine orange and ethidium bromide was obtained from (Promega, USA). Mueller Hinton agar, mannitol agar, Nutrient agar and Nutrient broth were procured from Salneca VOF Dutch technology (Netherlands), the Lysis Kit and the sequence of housekeeping gene are published (Abcam, USA).

Sample collection

Twelve swab samples from wounds and burns were collected in sterile tubes containing normal saline from patients of different hospitals for the isolation of *P. aeruginosa*, swabs samples were collected and transported aseptically to the laboratory within two hours of collection for the identification of the bacterial isolates¹⁴.

Bacterial Identification

Skin infection samples were immediately transferred into Mannitol agar which considered as selective medium for the isolation and identification of *S.aureus*. The isolates were examined for their shape, color. All plates were incubated at 37°C for 18-24 hours to identify the Bacterial types. The identified isolates were established with the Vitek system sourced by bioMérieux Vitek and specific conventional methods¹⁵.

Isolation and Identification of MRSA

This method was carried out by sub-culturing pure *Staphylococcus aureus* culture conserved on nutrient agar onto oxacillin resistant screening agar base (ORSAB) medium (Oxoid), and incubated at 37°C for 20 hours. The production of deep blue colonies indicates mannitol fermentation by isolates that are resistant to Oxacillin (or methicillin)¹⁶.

Characterizations of Silver nanoparticles

Transmission electron microscope (TEM)

TEM analysis was carried out by scanning the prepared zinc oxide nanoparticles to visualize the morphology and the size, Thin films of the sample were prepared on a Cover slide grid, the film on the TEM cover slide were allowed to dry by putting it at room temperature for a period and analysis it¹⁷.

Scanning electron microscope (SEM)

SEM examination was done to scan the silver nanoparticles to discern their morphology and size. A copper grid with carbon coating was used to prepare thin sample films. For this, very little amount of the sample was dropped on the grid, and a blotting paper was used to remove any extra solution; the film was dried by keeping the SEM grid under a mercury lamp for 5 min and then investigating it¹⁷.

Dynamic light scattering

Detection of light scattering from matter is a beneficial method with applications in several clinical disciplines wherein, relying at the light supply and detector, unique properties of molecules may be studied. One milliliter of distilled water turned into the cell after which 50 µl from stock dispersions have been added. Samples were once more sonicated for 5 mins. Length distributions of the nanoparticles had been identified through the DLS method.¹⁸

Antibacterial activity

Assessment of minimum inhibitory concentration

The minimum inhibitory concentration for against methicillin-resistant *Staphylococcus aureus* was determined. Briefly, the AgNPs were utilized at different concentration (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 µg / mL). Using standard wire loop, a loopful of methicillin-resistant *Staphylococcus aureus* culture in the examination with 0.5 McFarland standards, was inoculated into test tubes containing 1 mL of nutrient broth. These tubes were incubated at 37°C for 18-24 h and subsequently observed for development or turbidity¹⁹.

Agar well diffusion assay

The antibacterial activity of silver nanoparticles against methicillin *Staphylococcus aureus* was estimated by utilizing Muller Hinton agar. Methicillin-resistant *Staphylococcus aureus* isolates were spread on the surface of the Muller Hinton agar plate. Using a sterile tip, wells of 6 mm diameter were created on the agar. AgNPs were added to the wells at different concentrations (25, 50, 75, and 100 µg/mL). The plates with the test isolate were incubated for 24 h at 37 °C and later taken out to mark the inhibition zones.

The inhibition zone diameters were estimated, and for each isolate, the average value was calculated. All tests were performed in triplicates. To quantify the effect of silver nanoparticles in bacterial growth of methicillin *Staphylococcus aureus* curve .methicillin *Staphylococcus aureus* was cultured at 37°C on Muller-Hinton agar plates and culture from the new plates were used for inoculating 50 ml of nutrient broth medium. At that point, the bacterial growth was allowed till the (OD) reached 0.1 at 600nm, which represented to 10⁸ (CFU/ml). After that 1 ml of methicillin *Staphylococcus aureus* was added to 50 ml of nutrient broth culture media with AgNPs at concentration 50µg/mL and incubated on a shaker incubator at 37°C for 24 h. The growth of bacteria was evaluated by estimating optical density every 6 h using the spectrophotometer¹⁵.

SEM enabled bacterial morphology

The SEM was utilized for identification of morphological changes in methicillin *Staphylococcus aureus* when treated with AgNPs. The damage of the bacterial cell wall was envisioned by utilizing SEM. The methicillin *Staphylococcus aureus* treated with- and without AgNPs were centrifuged at 6000 rpm and washed 3 times utilizing PBS (50 mm, pH 7.3). Then, on a silicon wafer slide thin smear of the suspensions was spread, dried at room temperature and fixed with 1 mL of fixing buffer containing 0.1 M sucrose and 2.5 wt% glutaraldehyde with sodium cacodylate, and incubated for 1.5 h at 37°C. Following this, the samples fixed on SEM stubs were sputtered with gold for 5 min making a gold layer of 20 nm thickness to permit SEM perception. Bacterial morphology was contemplated utilizing a scanning electron (TESCAN, Vega III, Czech Republic)²⁰.

Acridine orange / Ethidium bromide double staining:

A fluorescence detecting instrument was utilized to detect the effectiveness of AgNPs on the nucleic acid of methicillin *Staphylococcus aureus*. To recognize live from dead cells, acridine orange fluoresces green (AO), and was used to stain live cells while ethidium bromide (EtBr), that fluoresces red was used to stain dead cells. In brief, 20 μL of bacterial strain (methicillin *Staphylococcus aureus* before and after treatment with silver nanoparticles were put in a microfuge tube and centrifuged for 15 min at 1500 rpm and washed with PBS three times. 5 μL of AO/EtBr were added and left for 5 min, then, a thin smear of treated methicillin *Staphylococcus aureus* was plated on a slide and inspected under a fluorescent microscope ²¹.

Quantitative Real-Time PCR (qRT-PCR) techniques

The expression study for azurin gene by Quantitative Real Time-PCR (qRT-PCR) method was done to assess the activity of AgNPs on mecA gene expression of the bacterial isolate. The primer sequence for amplification of this gene is:

Forward primer: GTGAAGATATACCAAGTGATT

Reverse primer: ATGCGCTATAGATTGAAAGGA

The RNA was extracted from the methicillin *Staphylococcus aureus* isolate using a commercial purification kit (Abcam ExCellenCT Lysis Kit). The purity of RNA was between 40 - 50 ng/ μL according to the RNA Extraction kit. Housekeeping gene primers for 16s rRNA gene was used as a control to measure the effect of gene expression. Detection of gene expression was done using ABM's One-Step BrightGreen qRT-PCR Kit ²².

Statistical analysis

The unpaired t-test with GraphPad Prism 6 was used statistical analysis of the acquired data. The values are presented as the Mean \pm SD of the three replicates of each experiment ²³.

RESULT AND DISCUSSION

Transmission electron microscope

To confirm and verify the physical (diameter, dispersion, morphology) parameters of the nanostructures, TEM had been employed. As seen in Figure (1), Silver nanoparticles had a colloidal morphology as targeted via the selection of the relative quantity fractions inside the synthesis of the constituent blocks within the diblock copolymer and had a particularly slim size distribution with diameter 23 and 30 nm.

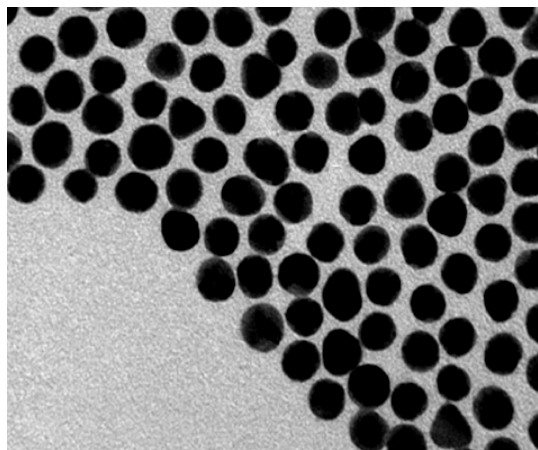


Figure 1: Transmission electron microscope of silver nanoparticles

The SEM images

The morphology and the dimensions of the silver nanoparticles were studied via scanning electron microscopy. Figure (1) exhibits the surface morphology of the silver nanoparticles (AgNPs). The SEM images show that the nanoparticle had spherical shape and the average size diameter of the AgNPs ranged from 20-30 nm. (Figure 2)

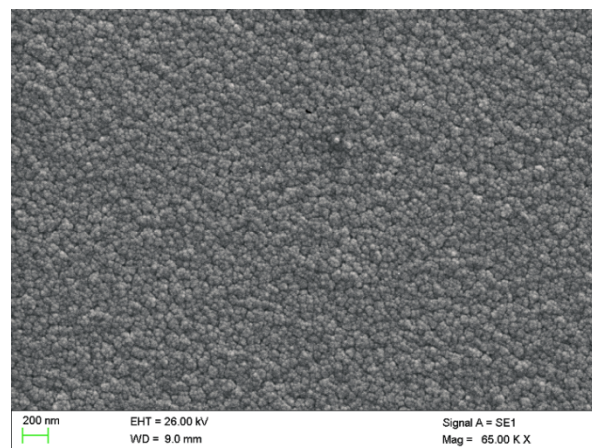


Figure 2: Scanning Electron Microscope of silver nanoparticles

Dynamic light scattering (DLS)

Dynamic light scattering technique is used to determine Brownian motion of spherical dispersed particles and to narrate this to the hydrodynamic length of the particles within the dispersed solution via dynamic fluctuations of scattered light intensity. This scattered light intensity is similarly mathematically manipulated to relate the hydrodynamic length of the debris. A vital characteristic of Brownian motion measured with the aid of DLS is that small debris circulates quicker in assessment to large debris, and the relationship between the dimensions of a particle and its velocity due to Brownian motion is defined within the Stokes-Einstein equation. As visualized in Fig. (3) Silver nanoparticles diameter has been within the range of 20-30 nm.

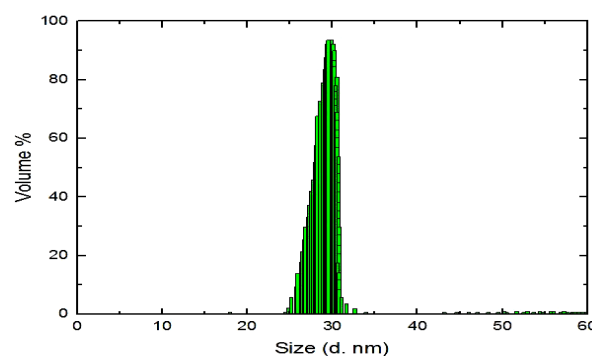


Figure 3: DLS histogram image of Silver nanoparticles

Assessment of minimum inhibitory concentration

The outcomes of this study confirmed that the AgNPs had activity against methicillin *Staphylococcus aureus* isolates in the range of 20 – 100 $\mu\text{g}/\text{mL}$. The minimum inhibitory concentration (MIC) of AgNPs on methicillin *Staphylococcus aureus* was seen at the concentration of 75 $\mu\text{g}/\text{mL}$. The outcomes of this study suggest that AgNPs represent antibacterial effect against Gram-positive (here, o methicillin *Staphylococcus aureus* bacterial isolates (24).

Agar well diffusion method

The outcomes of this method showed the antibacterial activity of AgNPs against methicillin *Staphylococcus aureus* as seen in Figure (4). The antibacterial activity of Silver nanoparticle at a concentration of 25 µg/ml⁻¹ against *S.aureus* exhibited inhibition zone with diameter (19 mm). While for 50 µg/ml⁻¹, the inhibition zone was (23 mm), Meanwhile 75 µg/ml⁻¹ against *S.aureus* exhibited inhibition zone with diameter (30 mm). While for 100 µg/ml⁻¹, the inhibition zone was (40 mm). The MIC was assessed to discern.

The lowest concentration that inhibited visible growth completely. The MIC of Ag-NPs against methicillin *Staphylococcus aureus* was 75 µg/ml⁻¹ as shown in Figure (4). At 75 µg/ml⁻¹ Ag-NPs concentration, the growth of bacterial strain was inhibited, however, when 50 µg/ml⁻¹ Ag-NPs was used, the growth of bacterial strain was only slightly inhibited²⁵.

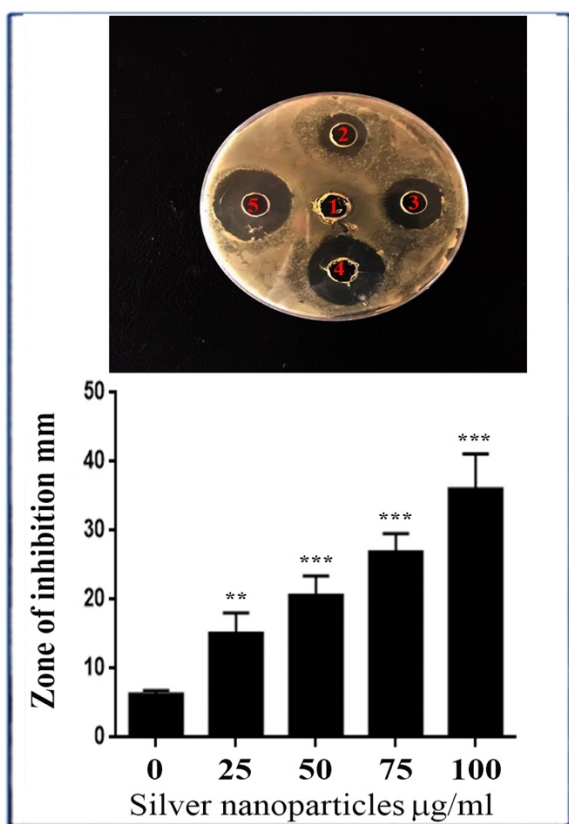


Figure 4: Anti-bacterial activity of Silver nanoparticles against methicillin *Staphylococcus aureus*.

1. Represented negative control, 2. Silver nanoparticles at concentration 25 µg/mL, 3. Silver nanoparticles at concentration 50 µg/mL, 4. Silver nanoparticles at concentration 75 µg/mL, 5. Silver nanoparticles at concentration 100 µg/mL the values are shown as the mean ± SD *p < 0.05, **p < 0.01, ***p < 0.001.

Particularly, the phenomenon of cell impermeability plays an important role in the resistance of bacteria. The mechanism of the bactericidal effect of silver nanoparticles remains to be illustrated. Smaller silver nanoparticles having a vast surface area accessible for interaction have a more prominent bactericidal effect than larger silver nanoparticles²⁶. It is additionally conceivable that silver nanoparticles not just interact with the surface of the membrane, but also penetrate inside the bacteria and inactivate DNA replicating capacity causing the desolation of the cell²⁷. In the current study, Increasing in concentration of Silver nanoparticles progressively inhibited the growth of bacterial isolate. The bacterial growth rate was measured using OD at

600nm at regular intervals as the increase bacterial number due to the growth would increase the OD values and this could reflect the inhibitory efficacy of Silver nanoparticles. To study the antibacterial activity of Silver nanoparticles, the bacterial isolates of methicillin *Staphylococcus aureus* were inoculated in nutrient broth with of Silver nanoparticles and without of Silver nanoparticles at concentration 50µg/ml. There was a clear inhibitory action of Silver nanoparticles especially after (6, 12, 18, 24) hr. of treated time Figure (5).

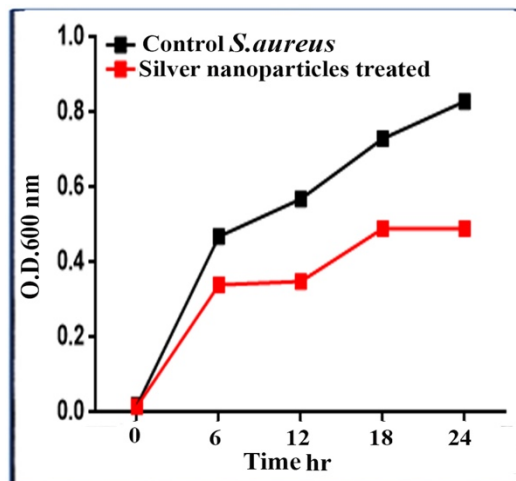


Figure 5: Effect of Ag nanoparticles in growth rate of methicillin *Staphylococcus aureus* shown as the mean±SD *p<0.001**

Bacterial morphology after treatment

The changes in methicillin *Staphylococcus aureus* cell morphological were examined through SEM (Fig. 4). The morphology of control cells was typically methicillin *Staphylococcus aureus* exist as a coccus shaped microorganism that generally occurs in grape-like clusters, but also could be seen as a singles and pairs with intact cell surface and no damage, while the bacterial cells treated with AgNPs at concentration 50 µg/mL underwent considerably minor structural changes as can be obviously discriminated in (Fig. 6). The SEM outcomes visualize the differences between the bacterial cells treated with AgNPs and non-treated bacterial cell. Interaction of the cell envelope with AgNPs from outside can create a disorder in cell membranes²⁸. Previous studies have shown the structural changes in the surface of bacteria after AgNPs treatment and suggest that the primary cause of cell death was the physical interaction with AgNPs^{29, 30}.

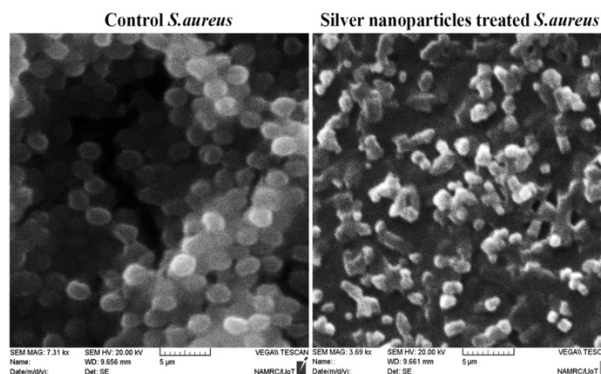


Figure 6: Visualization of methicillin *Staphylococcus aureus* treated with silver nanoparticles using SEM; the treated bacterial cells show damaged membrane

(Ahmed, Hashmi, Khan, & Musarrat, 2018)³¹ showed forceful antibacterial and antibiofilm activity against methicillin-

resistant *Staphylococcus aureus* because of the increased production of ROS and membrane permeability that lead to changed cell membrane morphology after treating methicillin-resistant *Staphylococcus aureus* with Ag Nps . These results confirm the role or effect of the Ag Nps on cell membrane of methicillin-resistant *Staphylococcus aureus* which were obvious cells under SEM.

Acridine orange / Ethidium bromide dual staining

The impact of tested AgNPs on the viability of methicillin-resistant *Staphylococcus aureus* isolates were studied using a fluorescent microscope in presence of stains: acridine orange/ethidium bromide (AO/EtBr). The outcomes from the double staining suggest that AgNPs shows a significantly high propensity to affect the bacterial cell membrane. As seen in figure (7), most of the cells appear in red color because of the interaction with AgNPs leading to damage and the loss of membrane integrity. DNA damage may occur through several distinct mechanisms. It can cause direct chemical damage to DNA by binding with and directly inducing double-strand breaks. Other studies report that AgNPs may interact in a manner with their target proteins that leads to DNA damage due to the inhibition of the ligase domain of topoisomerase, while the nuclease domains are left intact, thereby, allowing the enzyme to cleave DNA without re-ligation. This study proposes possible mechanisms underlying AgNPs-induced cytotoxicity in bacterial cells. First, AgNPs induce oxidative stress, causing instability in the cell membrane and making the membrane more permeable by incorporating Ag nanoparticles. This incorporation leads to the formation of pits on the membrane that is permeable, leading to a cellular osmotic breakdown, thus releasing the intracellular content.

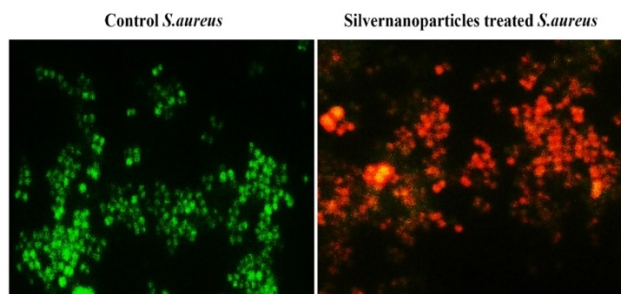


Figure 7: Fluorescence microscopic images of the green and red fluorescence stained methicillin *Staphylococcus aureus* in the absence and presence of Silver nanoparticles

Quantitative Real Time- PCR (qRT-PCR) techniques

The effective implication of AgNPs was estimated through *mecA* mRNA determination via qRT-PCR, using 16s rRNA gene as a control gene. As seen in Figure (8), their relative data of methicillin-resistant *Staphylococcus aureus mec a gene* which showed the downregulation of *mec a gene* of bacterial isolates that treated with Silver nanoparticle, that the fold change between treated and non-treated bacterial strains is approximately 1. Previous studies have reported the role of the *mec a gene* in antibiotic resistance such as methicillin, penicillin and other penicillin-like antibiotics. This *gene* does not allow the ring like structure of penicillin-like antibiotics to bind to the enzymes that help form the cell wall of the bacterium (transpeptidases), and hence the bacteria is able to replicate as normal(32). So the results reinforce the role of silver nanoparticles in inhibiting the efficiency of this gene.

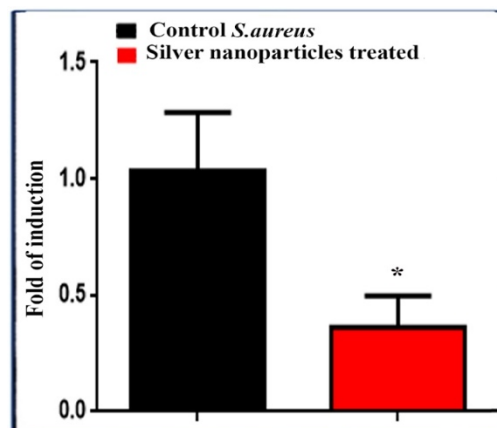


Figure 8: Silver nanoparticles down-regulates expression of Methicillin resistant *Staphylococcus aureus mec A gene*. The value are shown as the mean±SD *p<0.005

Due to the role in inhibiting growth via *mecA* gene, we can suggest that the AgNPs is a new generation of antimicrobial agent against the resistant microorganisms.

CONCLUSION

MRSA is a versatile, well-set pathogen with the potential to advance and adapt to its host as well as to the treatments changed to control its invasive damage. New treatments are required in the ongoing struggle. This results conclude that silver nanoparticles are remarkably effective in inhibiting the growth of bacterial isolates (here methicillin-resistant *Staphylococcus aureus*). Therefore, we can confirm that in future, the bactericidal effect of AgNPs can be applied as vital clinical and therapeutic agent, more so, because of the emergence of resistant microorganisms and a concurrent shortage of new antimicrobials. Therefore, silver nanoparticles can be effectively utilized as a powerful tool against multidrug-resistant bacteria.

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